Immunity

Supplemental Information

A Temporal Switch in the Germinal Center Determines Differential Output of Memory B and Plasma Cells

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SUPPLEMENTAL INFORMATION

Supplemental Data: Figures S1-S4

Supplemental Experimental Procedures

Supplemental References

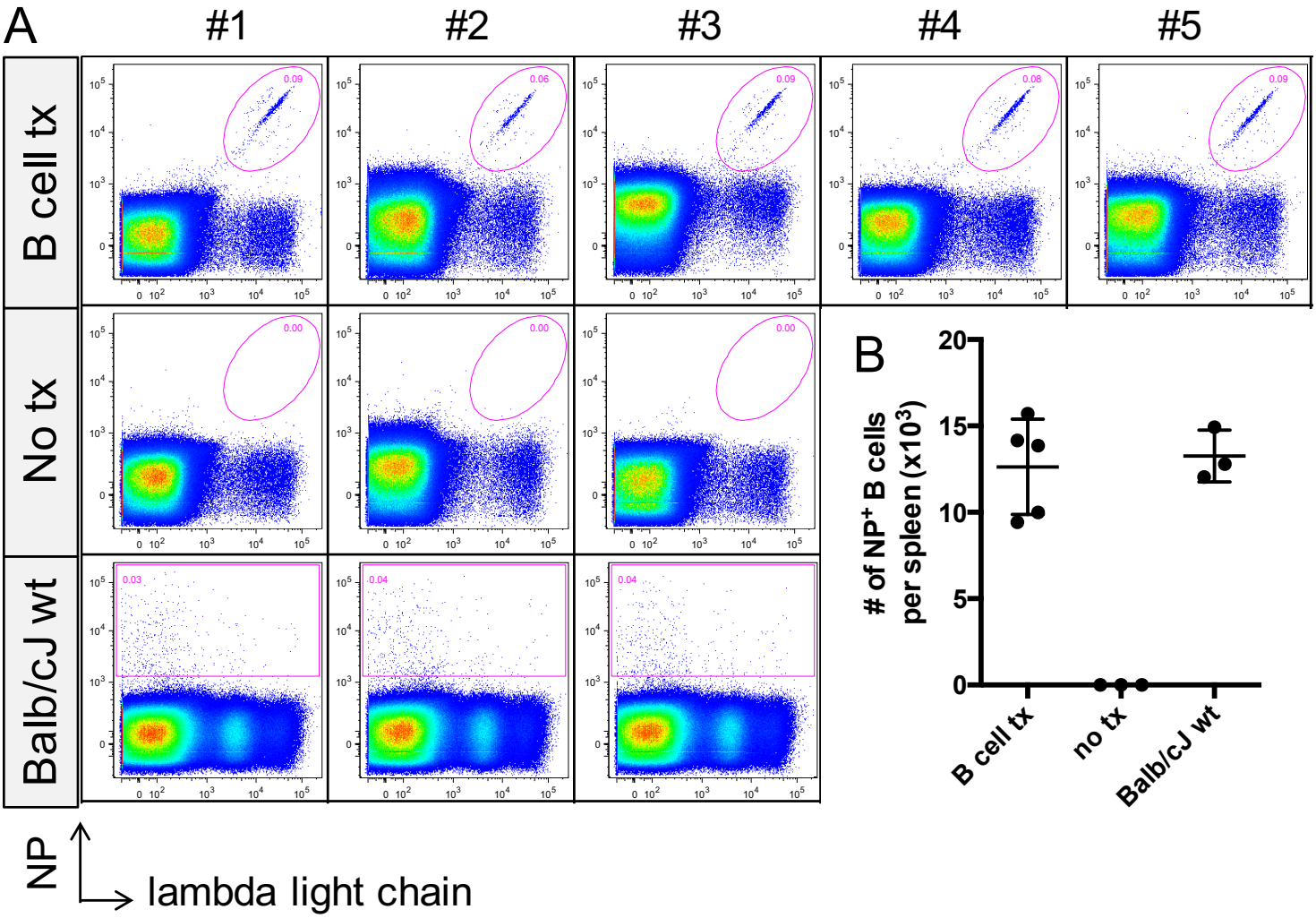

Figure S1, related to Fig. 1 and 2. Adoptive transfer system to adjust NP reactive precursor frequency to WT levels. AM14 transgenic x Vk8R genetically targeted BALB/cJ mice harbor an irrelevant monoclonal population of B cells and therefore are unable to mount endogenous NP-specific immune responses but otherwise display normal lymphoid architecture (Prak and Weigert, 1995; Shlomchik et al., 1993) with reduced numbers of B cells. These mice were adoptively transferred with an equivalent of $2x10^5$ NP reactive B cells from B1-8i$^{+/-}$ genetically targeted BALB/cJ mice, carrying a germline encoded, unmutated Vh186.2 site-directed transgene that encodes a BCR with moderate affinity for the hapten NP when paired with λ1 light chains (Sonoda et al., 1997), to allow donor B cell derived NP-specific immune responses. (**A**) 24h after adoptive transfer recipients were sacrificed and splenocytes were stained to determine the frequency of Igλ$^+$ NP-binding B cells. FACS plots are gated on live CD19$^+$ cells and each plot represents an individual AM14 transgenic x Vk8R genetically targeted BALB/cJ mouse either transferred with NP-reactive B cells (top row; B cell tx) or injected with transfer buffer only (middle row; No tx). Naive BALB/cJ WT mice served as control (bottom row). Numbers adjacent to outlined areas indicate percent gated population. (**B**) Quantification of frequencies of NP-reactive B cell displayed in (A) indicate a WT-like precursor frequency of on average of about $12.5x10^3$ NP-specific B cells per recipient spleen at the time point of immunization.

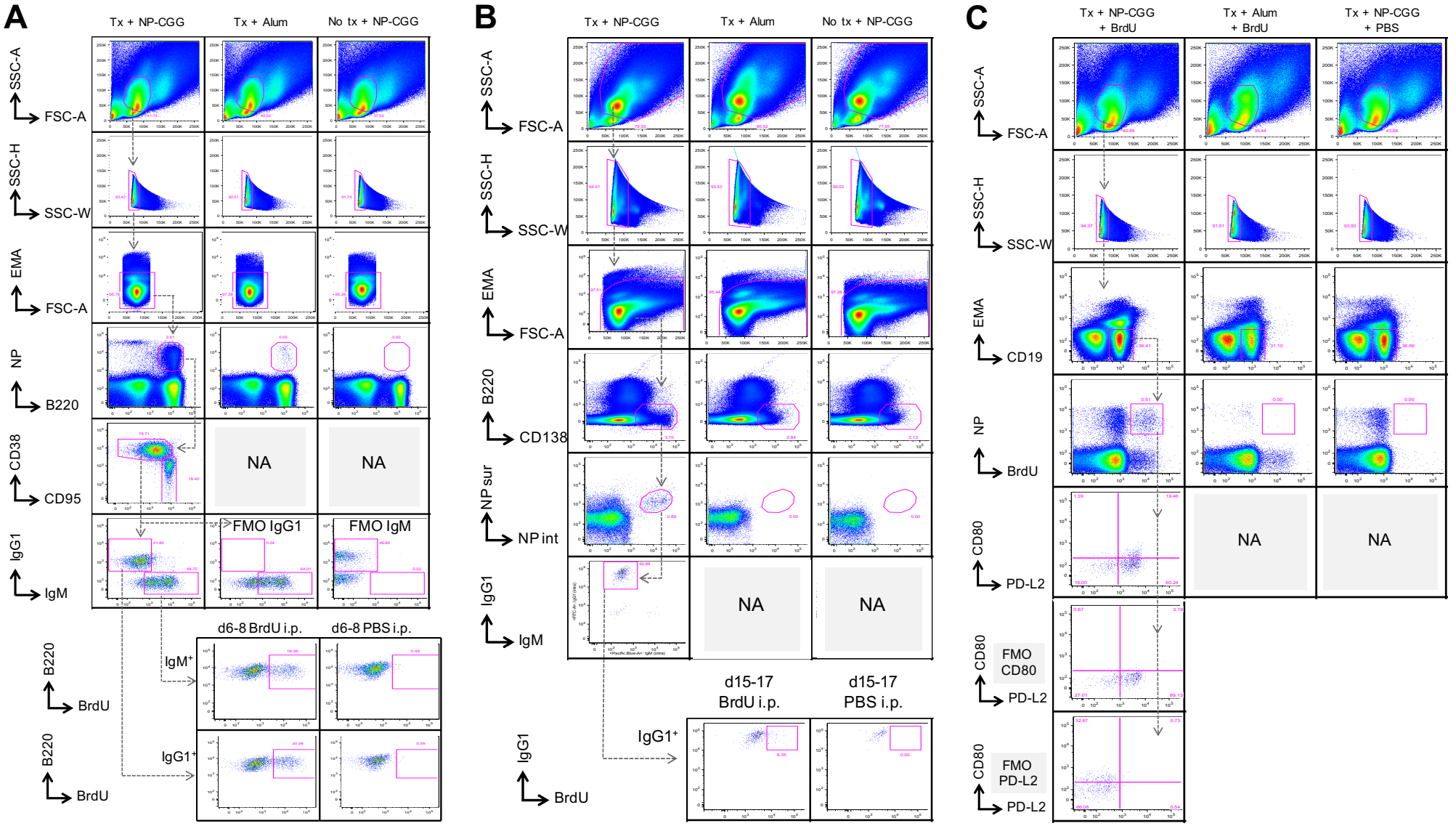

Figure S2, related to Fig. 1. Gating strategy for the detection of BrdU$^+$ MBC, MBC subsets and LLPC, 8 weeks after immunization of transfer recipients. Transfer recipients were immunized with NP-CGG in Alum (left column) or Alum only (middle column) 24h after NP-reactive B cell transfer and i.p. injected with BrdU or PBS at indicated time points as outlined in Fig. 1A. Mice immunized with NP-CGG in Alum without NP-specific B cell transfer (right column in A and B; no tx) served as controls. Single-cell suspensions of red blood cell-depleted splenocytes (A, C) or bone-marrow cells (B) were analyzed 8 weeks after immunization by flow cotometry to determine the frequency of BrdU$^+$ cells. (**A**) MBC were defined as EMA$^-$ NP$^+$ B220$^+$ CD38$^+$ CD95$^-$ and IgG1$^+$ or IgM$^+$ cells. (**B**) BM LLPC were stringently defined as EMA$^-$ B220$^-$ CD138$^+$ NP$^{surface-}$ NP$^{intracellular+}$ IgG1$^+$ cells. The distribution of CD80 and PD-L2 of EMA$^-$, NP$^+$ CD19$^+$ BrdU$^+$ cells was assessed in (**C**). Arrows indicate subsequent gating of populations and numbers next to outlined areas indicate percent gated population. Fluorescent minus one (FMO) controls were included to prove specificity of the Ab intentionally excluded from the staining solution. Not applicable (NA) is depicted instead of subsequent gating if parental gate contains virtually no cells.

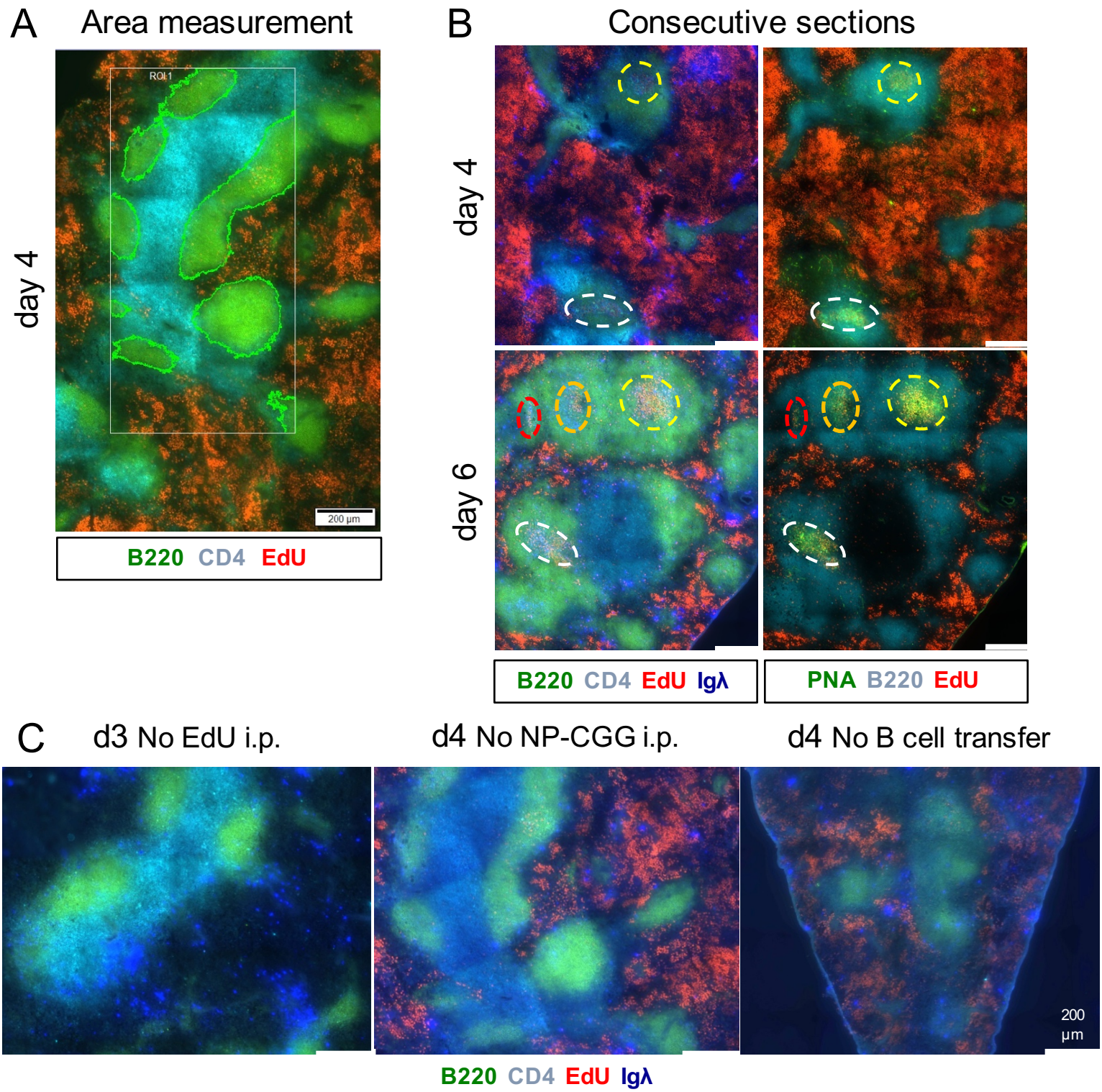

Figure S3, related to Fig. 3. Validation and quantification of histological analysis. Transfer recipients were injected with 0.85mg EdU at 6.5, 3.5 and 0.5h prior to harvesting spleens at indicated days post NP-CGG immunization. (**A**) Representative example of region definition and measurement. Regions of interest (ROI) were drawn around white pulps and the area of B cell zones was determined and measured by staining intensity using the count and measure analysis module of cellSens Dimensions software (Olympus). Areas of T cell zones where measured in analogous fashion (not depicted). Proliferating Ag specific ($Ig\lambda^+$ EdU^+) B cells were counted within each area. (**B**) Clustered proliferation of Ag specific B cells within B cell follicles (color-coded, dotted regions) was defined as GC reaction if counterstaining for peanut agglutinin (PNA) was positive on consecutive sections. The markers B220, EdU and Igλ (not depicted in right panel) were used to identify these regions on the consecutive sections (color-coded, dotted regions). Examples for d4 (upper panel) and d6 (lower panel) are shown. (**C**) Validation of specificity of measured response. Transfer recipients not given EdU injection (left panel) or without NP-CGG immunization (middle panel) or without B1-8 B cell transfer (right panel) did not show significant expansion of $Ig\lambda^+$ B cells. These controls indicate that the observed expansion of $Ig\lambda^+$ B cells was due to Ag-mediated activation of adoptively transferred B1-8 B cells. Scale bars are 200µm.

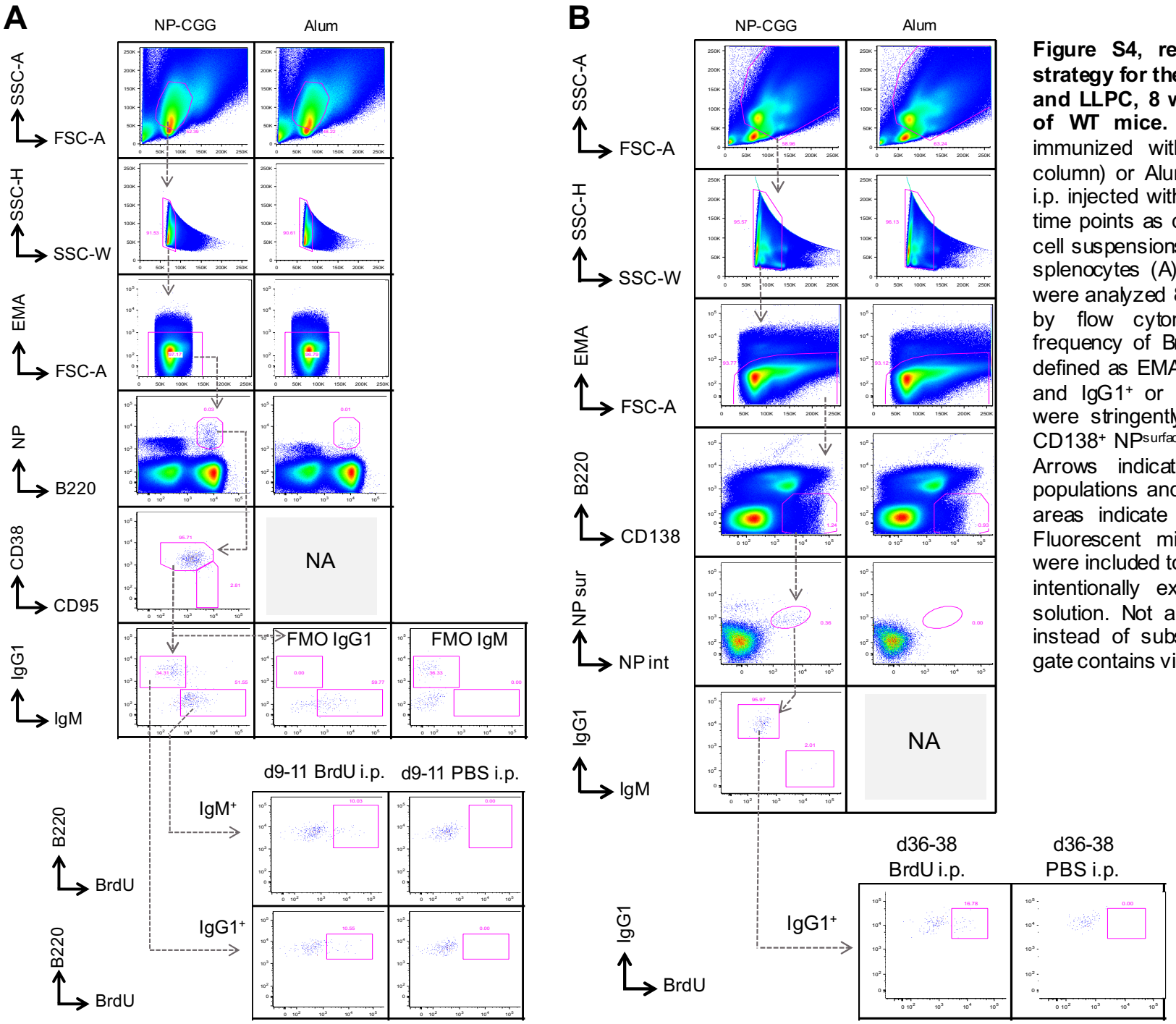

Figure S4, related to Fig. 4. Gating strategy for the detection of BrdU⁺ MBC and LLPC, 8 weeks after immunization of WT mice. BALB/cJ WT mice were immunized with NP-CGG in Alum (left column) or Alum only (right column) and i.p. injected with BrdU or PBS at indicated time points as depicted in Fig. 1A. Single-cell suspensions of red blood cell-depleted splenocytes (A) or bone marrow cells (B) were analyzed 8 weeks after immunization by flow cytometry to determine the frequency of BrdU⁺ cells. (**A**) MBC were defined as EMA⁻ NP⁺ B220⁺ CD38⁺ CD95⁺ and IgG1⁺ or IgM⁺ cells. (B) BM LLPC were stringently defined as EMA⁻ B220⁻ CD138⁺ $NP^{surface-}$ $NP^{intracellular+}$ IgG1⁺ cells. Arrows indicate subsequent gating of populations and numbers next to outlined areas indicate percent gated population. Fluorescent minus one (FMO) controls were included to prove specificity of the Ab intentionally excluded from the staining solution. Not applicable (NA) is depicted instead of subsequent gating if parental gate contains virtually no cells.

Antigens, antibodies and detection reagents

Chicken γ globulin (CGG; Sigma-Aldrich) or Bovine serum albumin (BSA) was haptenated with nitrophenyl (NP)-hydroxysuccinimide ester (Cambridge Research Biochemicals). Allophycocyanin, Phycoerythrin and BSA were haptenated with nitro-iodo-phenyl (NIP) – hydroxysuccinimide ester. The haptenation ratios of NP or NIP to proteins were determined by spectrometry. NP-33-CGG was used for immunizations. The following reagents were prepared and/ or conjugated in our laboratory: NIP_5-BSA-Alexa Fluor 680; anti-IgM (B7-6) Alexa Fluor 680 and Pacific Blue Ab; anti-CD19 (1D3.2) Pacific Blue or Alexa Fluor 647 Ab; anti-CD80 (16-10A1) Alexa Fluor 488 Ab; Anti-B220 (RA3-6B2) Alexa Fluor 488 or Alexa Fluor 647; anti-kappa (187.1) Pacific Blue; Alexa Fluor 647 or unconjugated anti-CD16/CD32 (2.4G2) Ab. Unconjugated polyclonal Goat anti-lambda Ab was purchased from Southern Biotech and conjugated to Alexa Fluor 680 and Pacific Blue in our laboratory. Unconjugated peanut agglutinin (PNA) was purchased from Vector Laboratories and conjugated to Alexa Fluor 488 in our laboratory. Anti-PDL2 (TY-25) biotin Ab, anti-CD38 (90) PE or biotin Ab were purchased from Biolegend. Anti-IgG1 (A85-1) FITC or V450 Ab and anti-CD138 (281-2) biotin or PE Ab were ordered from BD Biosciences. Anti-B220 (RA3-6B2) APC-Cy7 Ab, anti-CD19 (ID3) APC-Cy7 Ab, anti-CD95 (Jo2) Pe-Cy7 Ab and anti-CD73 (TY/23) PE Ab were from BD Pharmingen. Anti-BrdU (MoBU1) Alexa Fluor 647 Ab was from Invitrogen. Polyclonal anti-IgG1-AP Ab was purchased from Southern biotech. Ab-producing cell lines 174.2 (rat IgM anti-mouse CD4) and 31-68.1 (rat IgM anti- mouse CD8) were grown in RPMI 1640 media to generate antibody containing supernatants (Ceredig et al., 1985) for complement mediated cell depletion.

BrdU detection by multicolor flow cytometry

$1x10^7$ red blood cell depleted splenocytes were incubated with anti-CD16/CD32 Abs and EMA in staining buffer (SB; 1xPBS, 3%FCS, 2mM EDTA, 0.02% NaN_3) for 15min on ice followed by 10min exposure to fluorescent light. Cells were washed and stained for surface antigens for 30min on ice in SB and then washed in SB. Cell pellets were resuspended in 500µl cold 0.15M NaCl and 1.2ml of pre-cooled 100% ethanol were dropwise added while vortexing gently and incubated for 40min on ice. Cells were pelleted, washed once in SB and resuspended in 1ml 1% PFA supplemented with 0.05% Tween20 and incubated for 30min at RT followed by overnight incubation at 4°C. Cells were pelleted, washed once in SB and then resuspended in 1ml of 0.15M NaCl, 4.2mM $MgCl_2$ supplemented with 100Kunitz DNaseI (Sigma) for digestion of DNA. After 30min incubation at 37°C cells were pelleted and incubated for 20min on ice in 40µl SB with 10% rat- and 10% mouse serum (Equitech-bio, Inc) to block unspecific binding. 50µl SB with anti-BrdU Alexa Flour 647 Ab and any Abs for intracellular staining were added and incubated overnight at 4°C. Cells were washed twice in SB and analyzed at the LSRII cytometer.

EdU/ BrdU double detection by multicolor flow cytometry

$1x10^7$ red blood cell depleted splenocytes were incubated with anti-CD16/CD32 Abs and EMA in SB for 15min on ice followed by 10min exposure to fluorescent light. Cells were washed and surface-stained with anti-CD38 Alexa Fluor 680 Ab and anti-IgG1 V450 Ab for 30min at 4°C. Cells were washed once in SB followed by an additional washing step in 1xPBS supplemented with 1%BSA. Pellets were resuspended in 120µl fixative (component D of Click-it® EdU Flow cytometry assay kit, Invitrogen) and incubated for 20min at RT and 15min at 4°C. 3ml PBS/BSA were added and cells were pelleted. After one additional washing step in PBS/BSA cells were

resuspended in 2ml Perm/wash buffer (PW) of the click-it® EdU Flow cytometry assay kit and incubated for 5min at RT. Cells were pelleted and resuspended at a final volume of 80µl in PW. After 10min incubation at RT 400µl of the click-it reaction (prepared according to the manufacturers instructions) was added to the cells. After 40min incubation at RT cells were washed twice in PW followed by 2 additional washing steps in PBS/BSA and 2x SB. Cells were stained with NIP-PE and anti-CD95 PE-Cy7 Ab in SB for 30min at 4°C and then washed with 3ml SB. Cell pellets were resuspended in 500µl cold 0.15M NaCl and 1.2ml of pre-cooled 100% ethanol were dropwise added while vortexing gently and incubated for 40min on ice. Cells were pelleted, washed once in SB and resuspended in 1ml of 0.15M NaCl, 4.2mM $MgCl_2$ supplemented with 100Kunitz DNaseI. After 30min incubation at 37°C cells were pelleted and incubated for 20min on ice in 45µl SB with 10% rat and 10% mouse serum. 60µl SB with anti-B220 APC-Cy7 Ab and anti-BrdU Alexa Fluor 647 Ab were added and incubated overnight at 4°C. Cells were washed twice in SB and analyzed at the LSRII cytometer.

Gene expression profiling

mRNA was isolated from $3x10^5$ FACS Aria sorted cell with an RNeasy Micro kit (Qiagen). Biotinylated cRNA was generated with the Illumina TotalPrep RNA Amplification Kit (Life Technologies) and was hybridized to Illumina MouseWG-6 v2.0 Expression BeadChip arrays at the Yale Keck Microarray Facility. Data were analyzed with packages in software of the R project for statistical computing. Raw expression data were normalized by the quantile method provided by the lumi package in R/Bioconductor. Genes with different expression in the early versus late GC subset were defined by two criteria: an absolute difference in expression of ≥ 1.7, and a statistically significant change in expression as determined by false-discovery rate $q < 0.05$

(Alan Dabney and John D. Storey). qvalue: Q-value estimation for false discovery rate control. R package version 1.38.0.)

SUPPLEMENTAL REFERENCES

Ceredig, R., Lowenthal, J., Nabholz, M., and MacDonald, H. (1985). Expression of interleukin-2 receptors as a differentiation marker on intrathymic stem cells. Nature *314*, 98–100.

Prak, E.L., and Weigert, M. (1995). Light chain replacement: a new model for antibody gene rearrangement. J Exp Med *182*, 541–548.

Shlomchik, M.J., Zharhary, D., Saunders, T., Camper, S.A., and Weigert, M.G. (1993). A rheumatoid factor transgenic mouse model of autoantibody regulation. Int Immunol *5*, 1329–1341.

Sonoda, E., Pewzner-Jung, Y., Schwers, S., Taki, S., Jung, S., Eilat, D., and Rajewsky, K. (1997). B cell development under the condition of allelic inclusion. Immunity *6*, 225–233.